Divergent Effects of Anandamide Transporter Inhibitors with Different Target Selectivity on Social Play Behavior in Adolescent Rats

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ABSTRACT

The endocannabinoid system plays an important role in the modulation of affect, motivation, and emotion. Social play behavior is a natural reinforcer in adolescent rats, and we have recently shown that interacting endocannabinoid, opioid, and dopamine systems modulate social play. In the present study, we tested the hypothesis that, in contrast to administration of exogenous cannabinoid agonists, increasing local endocannabinoid signaling through anandamide transporter inhibition enhances social play. To this aim, we tested the effects of two anandamide transporter inhibitors with different target selectivity on social play behavior in adolescent rats. Interestingly, we found that the prototypical anandamide transporter inhibitor N-(4-hydroxyphenyl)-arachidonamide (AM404) reduced social play, whereas its more selective analog N-arachidonoyl-(2-methyl-4-hydroxyphenyl)amine (VDM11) enhanced it. The effects of AM404 were not mediated through its known pharmacological targets, since they were not blocked by the CB_1 cannabinoid receptor antagonist N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A), the CB_2 cannabinoid receptor antagonist N-(1,3,3-trimethylbicyclo(2.2.1)heptan-2-yl)-5-(4-chloro-3-methyl-phenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide (SR144528), or by the transient receptor potential vanilloid 1 receptor antagonist capsazepine. In contrast, the increase in social play induced by VDM11 was dependent on cannabinoid, opioid, and dopaminergic neurotransmission, since it was blocked by the CB_1 cannabinoid receptor antagonist SR141716A, the opioid receptor antagonist naloxone, and the dopamine receptor antagonist α-flupenthixol. These findings support the notion that anandamide plays an important role in the modulation of social interaction in adolescent rats, and they suggest that selective anandamide transporter inhibitors might be useful for the treatment of social dysfunctions. Furthermore, these results suggest that off-target effects may be responsible for some of the conflicting effects of anandamide transporter inhibitors on behavior.

Under physiological circumstances, brain CB_1 cannabinoid receptors are activated by lipid derivatives called endocannabinoids, including anandamide and 2-arachidonoylglycerol. Endocannabinoids are released on demand; that is, there is little, if any, tonic endocannabinoid signaling in the brain (Piomelli,

2003). Thus, the effects of exogenous cannabinoid receptor agonists, that lack the spatial and temporal specificity of locally released endocannabinoids, do not necessarily mimic the physiological functions of the endocannabinoid system. An alternative pharmacological approach to study the physiological and behavioral functions of endocannabinoid neurotransmission is to use indirect cannabinoid agonists, i.e., compounds that selectively interfere with endocannabinoid signaling by inhibiting either endocannabinoid synthesis or deactivation. These indirect (ant)agonists might be useful pharmacotherapeutic agents, since local modulation of endocannabinoid activity produces more subtle and selective effects than treatment with direct cannabinoid receptor agonists.

ABBREVIATIONS: CB, cannabinoid; FAAH, fatty acid amide hydrolase; URB597, cyclohexyl carbamic acid 3′-carbamoylbiphenyl-3-yl ester; WIN55,212-2, (2,3-dihydro-5-methyl-3-((4-morpholinyl)methyl)pyrrolo-(1,2,3-de)-1,4-benzoxazin-6-yl)(1-naphthalenyl)methanone monomethanesulfonate; AM404, N-(4-hydroxyphenyl)-arachidonamide; VDM11 (VDM), N-arachidonoyl-(2-methyl-4-hydroxyphenyl)amine; SR141716A (SR141), N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; SR144528, N-(1,3,3-trimethylbicyclo-(2.2.1)heptan-2-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide; TRPV1, transient receptor potential vanilloid 1; ANOVA, analysis of variance; CAP, capsazepine; NAL, naloxone; FLUP, α-flupenthixol; M-AEA, (R)-methanandamide.

Upon discovery of fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996), which catalyzes the intracellular hydrolysis of anandamide, several FAAH inhibitors were developed (Lambert and Fowler, 2005). For example, URB597 is a potent FAAH inhibitor that has no affinity for cannabinoid receptors and does not induce the well known effects of CB_1 cannabinoid receptor agonists, such as catalepsy, hypothermia, or hyperphagia (Kathuria et al., 2003). However, it exerts analgesic-, anxiolytic-, and antidepressant-like effects (Kathuria et al., 2003; Gobbi et al., 2005).

Given the involvement of the endocannabinoid system in affect, motivation, and emotion (Solinas et al., 2008), we recently started to investigate the role of endocannabinoids in social play behavior. Social play is the most characteristic and vigorous form of social interaction displayed by adolescent mammals, and it is crucial for social and cognitive development (Panksepp et al., 1984; Vanderschuren et al., 1997; Hol et al., 1999; van den Berg et al., 1999). Thus, animals housed in isolation during adolescence, when social play is most abundant, show several behavioral impairments in adulthood (Hol et al., 1999; van den Berg et al., 1999). Consistent with its importance for development, social play is a natural reinforcer (Humphreys and Einon, 1981; Calcagnetti and Schechter, 1992).

Interestingly, we found that the FAAH inhibitor URB597 increases social play behavior (Trezza and Vanderschuren, 2008a,b). The effect of URB597 on social play was exerted through neurotransmitter systems known to play key roles in positive emotions, i.e., cannabinoid, opioid, and dopaminergic neurotransmission. In contrast, the CB_1 cannabinoid receptor agonist WIN55,212-2 reduced social play behavior, through a nonopioid, nondopaminergic, CB_1 receptor-mediated mechanism. These results suggest that release of anandamide within the neural circuits mediating social behavior facilitates social play, whereas stimulating cannabinoid neurotransmission outside this circuitry may interfere with the normal execution of complex social acts (Trezza and Vanderschuren, 2008a,b). Moreover, these results highlight once more that direct and indirect cannabinoid agonists can have very different effects on behavior.

Before hydrolysis by intracellular FAAH can take place, anandamide needs to be transported into the cell (Beltramo et al., 1997; Glaser et al., 2005). Several ligands have been found to inhibit this process. In particular, AM404 is the best characterized anandamide uptake inhibitor in vivo. Its effects are comparable with those of URB597, i.e., it increases brain anandamide levels without the well known effects of CB_1 cannabinoid receptor agonists (Beltramo et al., 1997), and it has potentially beneficial properties for the treatment of pain, motor impairments, and anxiety disorders (Fernandez-Espejo et al., 2004; Bortolato et al., 2006; La Rana et al., 2006). These similarities led us to investigate whether URB597 and AM404 have comparable effects on social play behavior.

One important point of concern is that the behavioral effects of anandamide transporter inhibitors can be difficult to interpret. Some drugs currently available, including AM404, lack selectivity and have effects on noncannabinoid targets (Glaser et al., 2005; Lambert and Fowler, 2005). For these reasons, we compared the effects of AM404 on social play with those of its more selective analog VDM11 (De Petrocellis et al., 2000). In addition, we investigated the pharmacological mechanisms underlying the effects of these drugs on social play behavior.

Lack of cooperative play with peers and atypical social behavior are core symptoms of autism and antisocial personality disorders (American Psychiatric Association, 2000). By clarifying the role of endocannabinoid neurotransmission in the regulation of social behavior, the present findings may help to understand how the adolescent brain processes social information and may provide novel therapeutic targets for the treatment of developmental social dysfunctions. Furthermore, by investigating how target selectivity of anandamide transporter inhibitors influences behavioral responses, this study may aid in the development of more selective compounds to optimize the therapeutic opportunities offered by cannabinoid neurotransmission.

Materials and Methods

Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) arrived in our animal facility at 21 days of age and were housed in groups of four in $40 \times 26 \times 20$-cm (length $\times$ width $\times$ height) Macrolon cages under controlled conditions (20–21°C, 60–65% relative humidity, and 12/12-h light cycle with lights on at 7:00 AM). Food and water were available ad libitum.

All animals were experimentally naive and were used only once, with the following exception: the effects of AM404, VDM11, and (R)-$(+)$-arachidonyl-1′-hydroxy-2′-propyl amide) [(R)-methanandamide] on locomotor activity (Table 2), which have been widely described in the literature (de Lago et al., 2004; Bortolato et al., 2006), were assessed using animals that had been used previously as vehicle controls in a behavioral experiment. All experiments were approved by the Animal Ethics Committee of the University Medical Center Utrecht and were conducted in agreement with Dutch laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Drugs

The endocannabinoid uptake inhibitors AM404 and VDM11 (in bioavailable aqueous soya suspension (Tocrisolve; Tocris Bioscience, Avonmouth, UK) were diluted to the final concentration with saline. The stable anandamide analog (R)-methanandamide in absolute ethanol (Tocris Bioscience) was desiccated under a stream of nitrogen; the residue was then dissolved in a solution of 5% Tween 80 and 5% polyethylene glycol/saline. The CB_1 cannabinoid receptor antagonist SR141716A (National Institute of Mental Health's Chemical Synthesis and Drug Supply Program, National Institutes of Health, Bethesda, MD) and the CB_2 cannabinoid receptor antagonist SR144528 (National Institute of Mental Health's Chemical Synthesis and Drug Supply Program) were dissolved in 5% Tween 80 and 5% polyethylene glycol/saline. The TRPV1 vanilloid receptor antagonist capsazepine (Tocris Bioscience) was dissolved in 10% Tween 80 and 10% polyethylene glycol/saline. The opioid receptor antagonist naloxone (Tocris Bioscience) and the dopamine receptor antagonist α-flupenthixol (Sigma-Aldrich, Schnelldorf, Germany) were dissolved in saline. AM404 and (R)-methanandamide were given intraperitoneally 30 min before testing, whereas VDM11 (intraperitoneal) was administered 15 min before test. SR141716A (intraperitoneal), SR144528 (intraperitoneal), capsazepine (intraperitoneal), naloxone (subcutaneous), and α-flupenthixol (intraperitoneal) were given 30 min before AM404, (R)-methanandamide, or VDM11, respectively. Drug doses and pretreatment intervals were based on literature and on pilot experiments. In particular, we used the highest doses of SR141716A, capsazepine, naloxone, and α-flupenthixol that had no effect on social play by themselves (Table 1). Solutions were freshly prepared on the day of the experiment and were administered in a

TABLE 1
Effects of SR141 (intraperitoneal, 30 min before test), CAP (intraperitoneal, 1 h before test), NAL (subcutaneous, 30 min before test), and FLUP (intraperitoneal, 30 min before test) on pinning and pouncing frequencies
Data are mean ± S.E.M. frequency of pinning and pouncing ($n = 6–8$/group).

	Pinning		Pouncing	
Vehicle	30 ± 3		52 ± 4	
SR141, 0.1 mg/kg	30 ± 6	$F = 14.8, p < 0.001$	52 ± 7	$F = 15.5, p < 0.001$
SR141, 0.3 mg/kg	10 ± 2**		23 ± 3**	
SR141, 1 mg/kg	4 ± 1**		14 ± 3**	
Vehicle	26 ± 4		49 ± 8	
CAP, 10 mg/kg	29 ± 4	$F = 2.9, p = 0.08$	51 ± 6	$F = 2.4, p = 0.12$
CAP, 20 mg/kg	13 ± 3		25 ± 4	
Vehicle	31 ± 5		52 ± 6	
NAL, 0.3 mg/kg	21 ± 3	$F = 3.1, p < 0.05$	40 ± 6	$F = 4.4, p < 0.05$
NAL, 1 mg/kg	22 ± 5		36 ± 7	
NAL, 3 mg/kg	13 ± 2*		23 ± 4**	
Vehicle	35 ± 3		72 ± 5	
FLUP, 0.125 mg/kg	30 ± 6	$F = 8.2, p < 0.001$	60 ± 9	$F = 10.2, p < 0.001$
FLUP, 0.25 mg/kg	20 ± 4		44 ± 5*	
FLUP, 0.5 mg/kg	10 ± 3**		25 ± 6**	

** $p < 0.01$; * $p < 0.05$ (one-way ANOVA, followed by Tukey's post hoc test where appropriate).

volume of 2 ml/kg. Because of the importance of the neck area in the expression of social play behavior (Pellis and Pellis, 1987), subcutaneous injections were administered in the flank.

Procedures

Social Play Behavior. All the experiments were performed in a sound-attenuated chamber under dim light conditions. The testing arena consisted of a Plexiglas cage measuring $40 \times 40 \times 60$ cm (length × width × height), with approximately 2 cm of wood shavings covering the floor. The behavior of the animals was videotaped using a video camera with zoom lens, videotape recorder, and television monitor.

At 26 to 28 days of age, rats were individually habituated to the test cage for 10 min on each of the 2 days before testing. On the test day, the animals were socially isolated for 3.5 h before testing, to enhance their social motivation and thus facilitate the expression of social play behavior during testing. This isolation period has been shown to induce a half-maximal increase in the amount of social play behavior (Niesink and Van Ree, 1989). At the appropriate time before testing, pairs of animals were treated with drugs or vehicle. The test consisted of placing two similarly treated animals into the test cage for 15 min. The animals of each pair did not differ more than 10 g in body weight and had no previous common social experience.

Analysis from the videotape recordings was performed afterward. Coding of the drug solutions ensured that both during the experiment and analysis of behavior, the experimenter was unaware of the treatment of the animals. Behavior was assessed using the Observer 3.0 software (Noldus Information Technology B.V., Wageningen, The Netherlands).

In rats, a bout of social play behavior starts with one rat soliciting ("pouncing") another animal, by attempting to nose or rub the nape of its neck. The animal that is pounced upon can respond in different ways: if the animal fully rotates to its dorsal surface, "pinning" is the result, i.e., one animal lying with its dorsal surface on the floor with the other animal standing over it. From this position, the supine animal can easily initiate another play bout, by trying to gain access to the other animal's neck. Thus, during social play, pinning, which is considered to be the most obvious posture in social play behavior in rats, is not an endpoint but rather functions as a releaser of a prolonged play bout. If the animal that is pounced upon responds by evading, the soliciting rat may start to chase it, thus making another attempt to launch a play bout (Panksepp et al., 1984; Pellis and Pellis, 1987; Vanderschuren et al., 1997).

The following behaviors were scored per 15 min: frequency of pinning, frequency of pouncing, and time spent in social exploration, i.e., sniffing any part of the body of the test partner, including the anogenital area. Behaviors were scored per pair of animals, meaning that a test pair was treated as a single observation. Pinning is the result of an interaction between two animals; pouncing and social exploration were scored irrespective of which animal in a test pair performed the behavior.

Locomotor Activity. To assess whether the effects of AM404, VDM11, and (R)-methanandamide on social play were secondary to changes in locomotor activity, rats were tested, at 28 to 30 days of age, for horizontal locomotor activity in plastic cages ($50 \times 33 \times 40$ cm; length × width × height) using a videotracking system (Etho-Vision; Noldus Information Technology B.V.), which determined the position of the animal five times per second. At the appropriate time before testing, rats were treated with drugs or vehicle and then individually transferred from the home cage to the test cage, in which locomotor activity was monitored for 15 min.

Statistical Analysis

Behavior was analyzed per pair of animals. Thus, a pair of rats was treated as a single observation. Pinning and pouncing frequencies and time spent in social exploration were calculated per pair of animals and expressed as mean ± S.E.M. To assess the effects of single or combined treatments on social play behavior, data were analyzed using one-way or two-way ANOVA, respectively, followed by Tukey's post hoc test where appropriate. Horizontal locomotor activity was expressed as mean ± S.E.M. traveled distance (centimeters per 15 min). The effects of drug treatment on locomotor activity were analyzed with one-way ANOVA.

Results

Effects of AM404 on Social Play Behavior. The endocannabinoid uptake inhibitor AM404 dose-dependently decreased social play. At a dose of 5 mg/kg, it reduced pinning [$F_{3,31} = 6.19; p < 0.01$] (Fig. 1a) and pouncing [$F_{3,31} = 7.04; p = 0.001$] (Fig. 1b). The reduction in social play induced by AM404 was behaviorally specific and not secondary to a general loss in social interest. In fact, AM404 increased social exploration (Table 2), but this increase was not consistently observed in subsequent experiments. Furthermore, at the dose that affected social play, AM404 did not alter locomotor activity (Table 2). The CB$_1$ cannabinoid receptor antagonist SR141716A (0.1 mg/kg i.p.) did not block the reduction in pinning [$F_{(SR141)1,28} = 2.73$, n.s; $F_{(AM)1,28} = 22.53, p < 0.001$; $F_{(SR141xAM)1,28} = 0.25$, n.s.] (Fig. 2a) and pouncing [$F_{(SR141)1,28} = 3.3$, n.s.; $F_{(AM)1,28} = 34.73, p < 0.001$; $F_{(SR141xAM)1,28} = 0.13$, n.s.] (Fig. 2b) induced by AM404 (5 mg/kg i.p.). Post hoc analysis showed that AM404 reduced
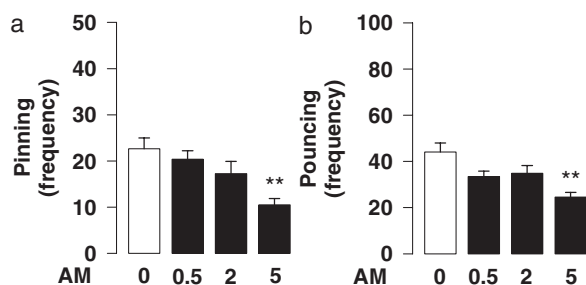
Fig. 1. Effects of the anandamide uptake inhibitor AM404 on social play behavior. AM404 (AM; 0.5, 2, and 5 mg/kg i.p., 30 min before test) dose-dependently reduced pinning (a) and pouncing (b). Data represent mean ± S.E.M. frequency of pinning and pouncing. **, $p < 0.01$ versus vehicle (i.e., 0 mg/kg AM404; Tukey's post hoc test; $n = 8$/group).

pinning and pouncing in both vehicle- and SR141716A-pretreated rats. Furthermore, SR141716A did not alter the effect of AM404 on social exploration [$F_{(SR141)1,28} = 3.37$, n.s; $F_{(AM)1,28} = 15.94$, $p < 0.001$; $F_{(SR141xAM)1,28} = 0.28$, n.s.] (data not shown). These data show that CB_1 cannabinoid receptors are not involved in the effects of AM404 on social play. Since CB_2 cannabinoid receptors have been recently described in brain regions involved in emotion, motivation, and affect (Van Sickle et al., 2005), we tested whether CB_2 cannabinoid receptors were involved in the effects AM404 on social play. Pretreatment with the CB_2 cannabinoid receptor antagonist SR144528 (0.1 mg/kg i.p.) did not counteract the inhibitory effect of AM404 on social play (pinning: [$F_{(SR144)1,23} = 0.00028$, n.s.; $F_{(AM)1,23} = 22.32$, $p < 0.001$; $F_{(SR144xAM)1,23} = 0.002$, n.s.], Fig. 2c; and pouncing: [$F_{(SR144)1,23} = 0.072$, n.s; $F_{(AM)1,23} = 17.80$, $p < 0.001$; $F_{(SR144xAM)1,23} = 0.012$, n.s.], Fig. 2d). Post hoc analysis showed that AM404 reduced pinning and pouncing in both vehicle- and SR144528-pretreated rats. Besides inhibition of endocannabinoid uptake, AM404 also activates a ligand-activated cation channel called TRPV1 vanilloid receptor (Zygmunt et al., 2000). We therefore also tested whether TRPV1 vanilloid receptors were involved in the effects of AM404 on social play. However, neither the TRPV1 vanilloid receptor antagonist capsazepine (10 mg/kg i.p.; pinning: [$F_{(CAP)1,38} = 4.28$, $p < 0.05$; $F_{(AM)1,38} = 23.99$, $p < 0.001$; $F_{(CAPxAM)1,38} = 0.24$, n.s.], Fig. 2e; and pouncing: [$F_{(CAP)1,38} = 2.42$, n.s.; $F_{(AM)1,38} = 23.37$, $p < 0.001$; $F_{(CAPxAM)1,38} = 0.58$, n.s.], Fig. 2f) nor combined treatment with SR141716A (0.1 mg/kg i.p.) and capsazepine (10 mg/kg i.p.; pinning: [$F_{(SR141+CAP)1,28} = 0.07$, n.s.; $F_{(AM)1,28} = 34.08$, $p < 0.001$; $F_{(SR141+CAPxAM)1,28} = 0.33$, n.s.], Fig. 2g; and pouncing: [$F_{(SR141+CAP)1,28} = 0.67$, n.s.; $F_{(AM)1,28} = 30.75$, $p < 0.001$; $F_{(SR141+CAPxAM)1,28} = 0.08$, n.s.], Fig. 2h) blocked the effects of AM404 on social play. Post hoc analysis showed that AM404 reduced pinning and pouncing in vehicle-, capsazepine- and SR141716A- plus capsazepine-pretreated rats. Together, these results show that the effects of AM404 on social play were not mediated by activation of the well known pharmacological targets of this drug, i.e., CB_1 and CB_2 cannabinoid and TRPV1 vanilloid receptors. Furthermore, these results rule out the possibility that redundant binding of anandamide to either CB_1 cannabinoid or TRPV1 vanilloid receptors underlies the reduction in social play induced by AM404.

We have previously shown that the increase in social play induced by the FAAH inhibitor URB597 involves opioid and dopaminergic neurotransmission, since it was blocked by the opioid receptor antagonist naloxone and the dopamine receptor antagonist α-flupenthixol (Trezza and Vanderschuren, 2008a). However, neither naloxone (1 mg/kg s.c.; pinning: [$F_{(NAL)1,29} = 0.11$, n.s.; $F_{(AM)1,29} = 28.45$, $p < 0.001$; $F_{(NALxAM)1,29} = 0.081$, n.s.], Fig. 2j; and pouncing: [$F_{(NAL)1,29} = 0.24$, n.s.; $F_{(AM)1,29} = 44.08$, $p < 0.001$; $F_{(NALxAM)1,29} = 0.56$, n.s.], Fig. 2k) nor α-flupenthixol (0.125 mg/kg i.p.; pinning: [$F_{(FLUP)1,29} = 0.34$, n.s.; $F_{(AM)1,29} = 23.14$, $p < 0.001$; $F_{(FLUP xAM)1,29} = 0.05$, n.s.], Fig. 2l; and pouncing: [$F_{(FLUP)1,29} = 2.51$, n.s.; $F_{(AM)1,29} = 23.65$, $p < 0.001$; $F_{(FLUPxAM)1,29} = 0.22$, n.s.], Fig. 2m) blocked the reduction in social play induced by AM404 (5 mg/kg i.p.). Post hoc analysis showed that AM404 reduced pinning and pouncing in rats pretreated with vehicle, naloxone, and α-flupenthixol.

Effects of VDM11 on Social Play Behavior. VDM11 increased pinning [$F_{2,37} = 4.66$; $p < 0.05$] (Fig. 3a) and pouncing [$F_{2,37} = 6.88$; $p < 0.01$] (Fig. 3b) at doses of 0.5 and 1 mg/kg. Social exploration and locomotor activity were not affected by VDM11 (Table 2). The increase in pinning [$F_{(SR)1,43} = 6.67$, $p < 0.05$; $F_{(VDM)1,43} = 4.78$, $p < 0.05$; $F_{(SRxVDM)1,43} = 8.01$, $p < 0.01$] (Fig. 4a) and pouncing [$F_{(SR)1,43} = 10.08$, $p < 0.01$; $F_{(VDM)1,43} = 7.82$, $p < 0.01$; $F_{(SRxVDM)1,43} = 9.30$, $p < 0.01$] (Fig. 4b) induced by VDM11 (0.5 mg/kg i.p.) was blocked by the CB_1 cannabinoid receptor antagonist SR141716A, at a dose that by itself had no effects on social play behavior (0.1 mg/kg i.p.; Table 1). Post hoc analysis showed that VDM11 increased pinning and pouncing in vehicle-, but not in SR141716A-pretreated animals, indicating that the effects of VDM11 on social play were mediated by activation of CB_1 cannabinoid receptors.

Subsequently, we investigated whether the effects of VDM11 on social play behavior also involved activation of opioid and dopaminergic neurotransmission. Pretreatment with a dose of naloxone that had no effect on social play by itself (1 mg/kg s.c.; Table 1) prevented the effect of VDM11 (0.5 mg/kg i.p.) on pinning [$F_{(NAL)1,43} = 9.58$, $p < 0.01$; $F_{(VDM)1,43} = 4.70$, $p < 0.05$; $F_{(NALxVDM)1,43} = 3.14$, $p = 0.08$] (Fig. 5a) and pouncing [$F_{(NAL)1,43} = 5.50$, $p < 0.05$; $F_{(VDM)1,43} = 19.01$, $p < 0.001$; $F_{(NALxVDM)1,43} = 1.84$, $p < 0.001$] (Fig. 5b). Post hoc analysis revealed that VDM11 increased pinning and pouncing in vehicle-, but not in naloxone-pretreated animals, demonstrating that stimulation of opioid receptors is involved in the effect of VDM11 on social play. At a dose that had no effect on social play behavior by itself, the dopamine receptor antagonist α-flupenthixol (0.125 mg/kg i.p.; Table 1) blocked the effect of VDM11 (0.5 mg/kg i.p.) on pinning [$F_{(FLUP)1,43} = 9.73$, $p < 0.001$; $F_{(VDM)1,43} = 3.61$, $p = 0.06$; $F_{(FLUPxVDM)1,43} = 8.72$, $p < 0.01$] (Fig. 5c) and pouncing [$F_{(FLUP)1,43} = 9.75$, $p < 0.01$; $F_{(VDM)1,43} = 11.67$, $p < 0.01$; $F_{(FLUPxVDM)1,43} = 7.84$, $p < 0.01$] (Fig. 5d). Post hoc analysis showed that VDM11 increased pinning and pouncing in vehicle- but not in α-flupenthixol-pretreated rats. Together, these results show that, similar to the FAAH inhibitor URB597, the effects of VDM11 on social play depend on stimulation of opioid and dopamine receptors.

Effects of (R)-Methanandamide on Social Play Behavior. To compare the effects of indirect versus direct activation of CB_1 cannabinoid receptors by anandamide on social play behavior, we tested the hydrolytically stable analog of anandamide, (R)-methanandamide. (R)-Methanandamide decreased social play, since it reduced pinning [$F_{3,31} = 3.98$; $p < 0.05$] (Fig. 6a) at the dose of 3 mg/kg and pouncing [$F_{3,31} = 7.79$; $p < 0.001$] (Fig. 6b) at the doses of 0.3 and 3

TABLE 2
Social exploration and locomotor activity after treatment with AM404 (5 mg/kg, 30 min before test), VDM11 (0.5 mg/kg, 15 min before test), and (R)-methanandamide (3 mg/kg, 30 min before test)

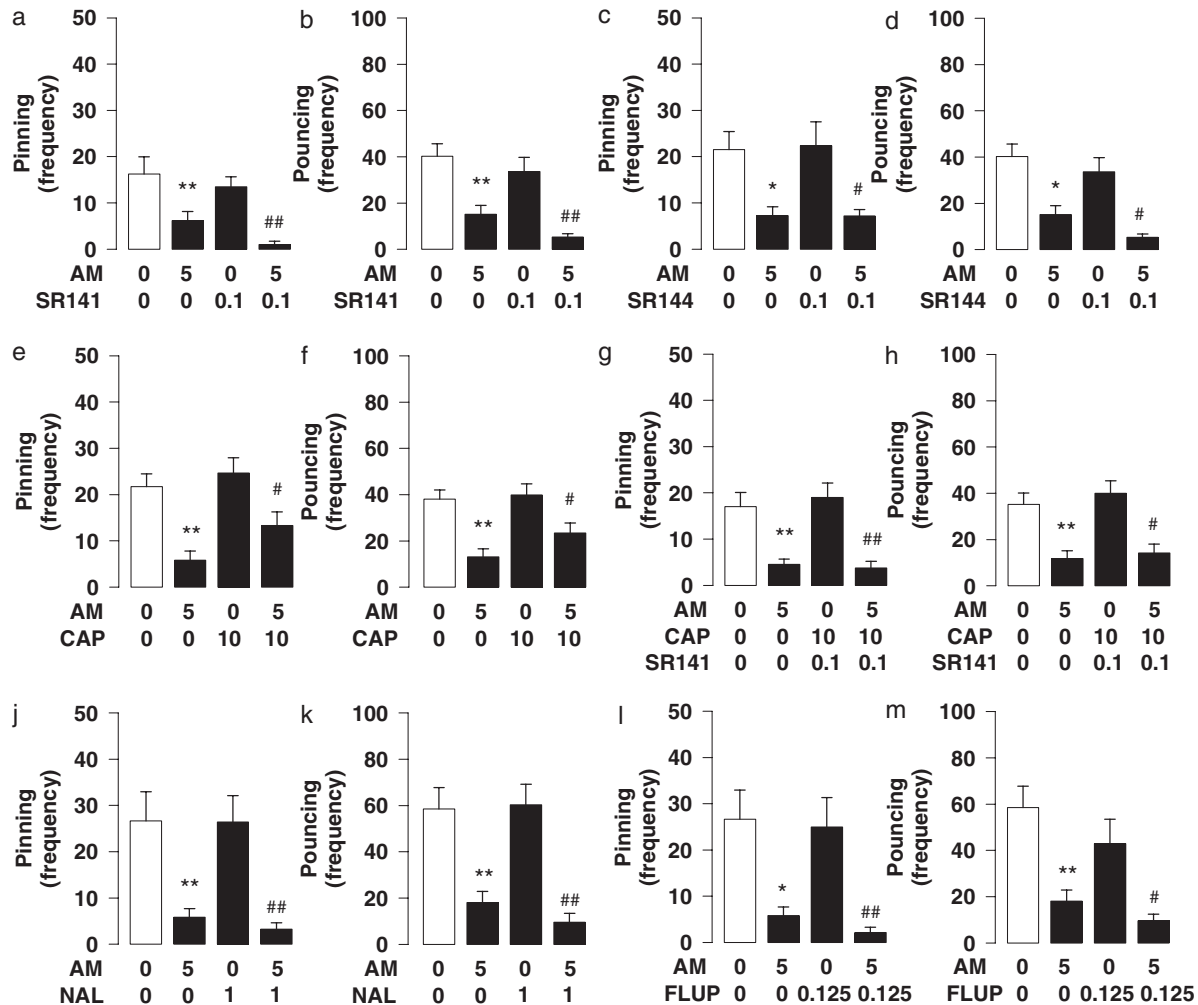|	Social Exploration (s/15 min)		Traveled Distance (cm/15 min)	
Vehicle	39 ± 6	$F = 5.1, p < 0.01$	1570 ± 188	$F = 0.09, p = 0.76$
AM404	$84 \pm 11^*$		1502 ± 115	
Vehicle	41 ± 4	$F = 1.8, p = 0.18$	2080 ± 257	$F = 1.1, p = 0.31$
VDM11	55 ± 7		2380 ± 122	
Vehicle	28 ± 2	$F = 0.07, p = 0.97$	1887 ± 505	$F = 2.13, p = 0.17$
(R)-Methanandamide	29 ± 3		1547 ± 150	

$* p < 0.05$ (ANOVA).

Fig. 2. Effects of AM404 (AM; 5 mg/kg i.p., 30 min before test) on pinning (a and c) and pouncing (b and d) were not blocked by the CB$_1$ cannabinoid receptor antagonist SR141716A (SR141; 0.1 mg/kg i.p., 30 min before AM404 (a and b) or by the CB$_2$ cannabinoid receptor antagonist SR144528 (SR144; 0.1 mg/kg i.p., 30 min before AM404 (c and d). Furthermore, neither the TRPV1 vanilloid receptor antagonist capsazepine (CAP; 10 mg/kg, i.p., 30 min before AM404 (e and f) nor combined treatment with SR141716A (SR141; 0.1 mg/kg i.p., 30 min before AM404) and capsazepine (CAP; 10 mg/kg i.p., 30 min before AM404; (g and h) blocked the effects of AM404 on social play. AM404 also reduced pinning (j and l) and pouncing (k and m) after pretreatment with the opioid receptor antagonist naloxone (NAL; 1 mg/kg s.c., 30 min before AM404 (j and k) and the dopamine receptor antagonist α-flupenthixol (FLUP; 0.125 mg/kg i.p., 30 min before AM404 (l and m). Data represent mean ± S.E.M. frequency of pinning and pouncing. *, $p < 0.05$ and **, $p < 0.01$ versus vehicle (i.e., 0 mg/kg AM404 plus 0 mg/kg SR141716A/SR144528/capsazepine/naloxone/α-flupenthixol). #, $p < 0.05$ and ##, $p < 0.01$ versus SR141716A alone (a and b), SR144528 alone (c and d), capsazepine alone (e and f), capsazepine plus SR141716A (g and h), naloxone alone (j and k), or flupenthixol alone (l and m) (Tukey's post hoc test; $n = 7$–13/group).

mg/kg. These effects were behaviorally specific, since (R)-methanandamide did not alter social exploratory behavior or locomotor activity (Table 2).

The reduction in pinning [$F_{(SR)1,28} = 0.018$, n.s.; $F_{(M\text{-}AEA)1,28} = 8.01, p < 0.01$; $F_{(SRxM\text{-}AEA)1,28} = 1.58$, n.s.] (Fig. 6c) and pouncing [$F_{(SR)1,28} = 0.62$, n.s.; $F_{(M\text{-}AEA)1,28} = 5.62, p < 0.05$; $F_{(SRxM\text{-}AEA)1,28} = 3.12, p = 0.08$] (Fig. 6d) induced

by (R)-methanandamide (3 mg/kg i.p.) was blocked by the CB$_1$ cannabinoid receptor antagonist SR141716A. Post hoc analysis showed that (R)-methanandamide reduced pinning and pouncing in vehicle-, but not in SR141716A-pretreated animals, demonstrating that the effects of (R)-methanandamide on social play were mediated by activation of CB$_1$ cannabinoid receptors.
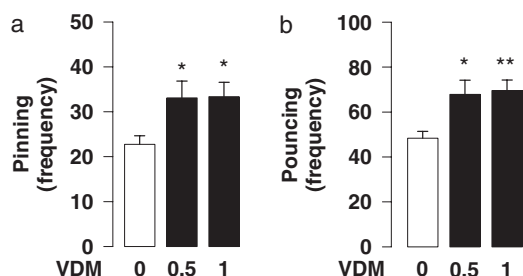
Fig. 3. Effects of the anandamide uptake inhibitor VDM11 on social play behavior. VDM11 (VDM; 0.5–1 mg/kg i.p., 15 min before test) enhanced pinning (a) and pouncing (b). Data represent mean ± S.E.M. frequency of pinning and pouncing. *, $p < 0.05$ and **, $p < 0.01$ versus vehicle (i.e., 0 mg/kg VDM11; Tukey's post hoc test; $n = 12$–15/group).
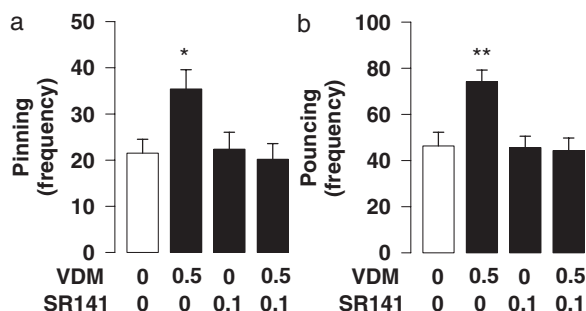

Fig. 4. Effects of VDM11 (VDM; 0.5 mg/kg i.p., 15 min before test) on pinning (a) and pouncing (b) were mediated by activation of CB_1 cannabinoid receptors, since they were blocked by the CB_1 cannabinoid receptor antagonist SR141716A (SR141; 0.1 mg/kg i.p., 30 min before VDM11). Data represent mean ± S.E.M. frequency of pinning and pouncing. *, $p < 0.05$ and **, $p < 0.01$ versus vehicle (i.e., 0 mg/kg VDM 11 plus 0 mg/kg SR141716A; Tukey's post hoc test; $n = 12$/group).

Discussion

Anandamide transporter inhibitors represent a novel class of positive modulators of endocannabinoid neurotransmission. These compounds are useful pharmacological tools to unravel the physiological functions of endocannabinoid signaling. In addition, these drugs also hold promise from a clinical perspective, because they can enhance endocannabinoid signaling, but lack the adverse effects of direct cannabinoid receptor agonists such as catalepsy and hypothermia. In the present study, we used anandamide uptake inhibitors with different target selectivity to investigate the role of endocannabinoid neurotransmission in social play behavior in adolescent rats.

We found that anandamide uptake inhibitors with different target selectivity have divergent effects on social play behavior. The prototypical anandamide uptake inhibitor AM404 reduced social play, whereas its more selective analog VDM11 enhanced it. The effects of AM404 on social play were behaviorally specific: AM404 did not alter locomotor activity, and it slightly increased social exploration in some experiments but did not affect it in others. AM404 was the first-developed synthetic inhibitor of anandamide uptake (Beltramo et al., 1997). It enhances CB_1 cannabinoid receptor-mediated anandamide responses both in vitro and in vivo, without mimicking the typical effects of CB_1 cannabinoid receptor agonists, such as catalepsy and hypothermia (Beltramo et al., 1997, 2000; Fegley et al., 2004). The doses of AM404 used in the present study have been shown to increase anandamide levels in several brain areas (Bortolato et al., 2006). How-
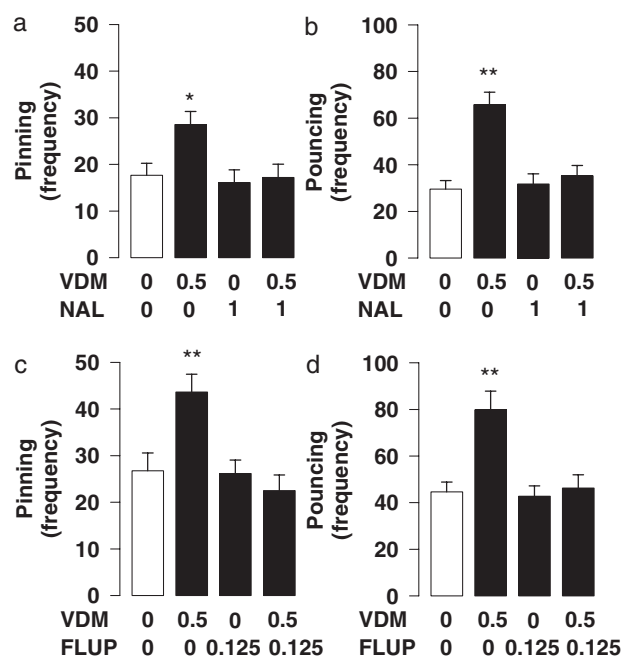

Fig. 5. Effects of VDM11 (VDM; 0.5 mg/kg i.p., 15 min before test) on pinning (a and c) and pouncing (b and d) were blocked by the opioid receptor antagonist NAL (1 mg/kg s.c., 30 min before VDM11) (a and b) and by the dopamine receptor antagonist FLUP (0.125 mg/kg i.p., 30 min before VDM11) (c and d). Data represent mean ± S.E.M. frequency of pinning and pouncing. *, $p < 0.05$ and **, $p < 0.01$ versus vehicle (i.e., 0 mg/kg VDM11 plus 0 mg/kg naloxone/α-flupenthixol; Tukey's post hoc test; $n = 12$/group).
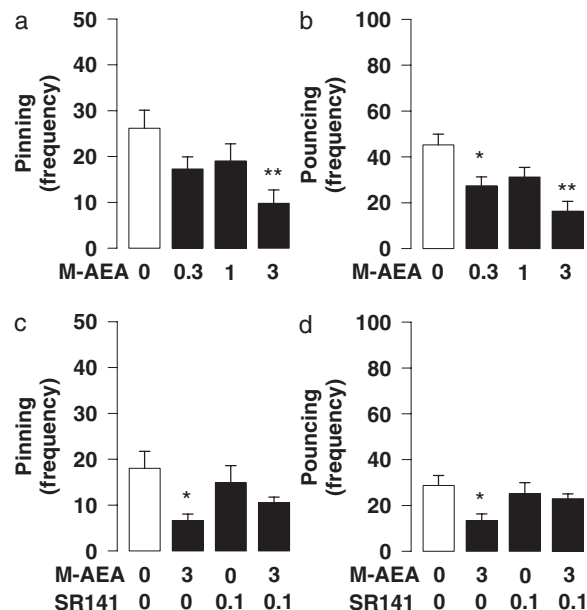

Fig. 6. Effects of (R)-methanandamide on social play behavior. (R)-methanandamide (M-AEA; 0.3–1-3 mg/kg i.p., 30 min before test) dose-dependently reduced pinning (a) and pouncing (b). The effects of (R)-methanandamide (M-AEA; 0.3 mg/kg i.p., 30 min before test) on pinning (c) and pouncing (d) were blocked by the CB_1 cannabinoid receptor antagonist SR141716A (SR141; 0.1 mg/kg i.p., 30 min before M-AEA). Data represent mean ± S.E.M. frequency of pinning and pouncing. *, $p < 0.05$ and **, $p < 0.01$ versus vehicle [i.e., 0 mg/kg (R)-methanandamide (a and b); 0 mg/kg (R)-methanandamide plus 0 mg/kg SR141716A (c and d); Tukey's post hoc test; $n = 8$/group].

ever, the effects of this drug on social play were not blocked by the CB_1 cannabinoid receptor antagonist SR141716A at a dose that blocks the effects of the FAAH inhibitor URB597 on

social play, nor by the CB_2 receptor antagonist SR144528, suggesting that they were not mediated by increased anandamide signaling at CB_1 or CB_2 cannabinoid receptors.

In addition to its inhibitory effects on anandamide transport, AM404 directly interacts with noncannabinoid pharmacological targets (Glaser et al., 2005). In particular, it is a full agonist at TRPV1 vanilloid receptors, with affinity and efficacy at least 10-fold higher than for the anandamide transporter (De Petrocellis et al., 2000; Zygmunt et al., 2000). TRPV1 vanilloid receptors are cation channels primarily involved in the integration of noxious stimuli in peripheral sensory nerve terminals (Szallasi and Di Marzo, 2000). They have also been found in several brain areas, where they might be involved in the modulation of synaptic plasticity (Mezey et al., 2000). Anandamide binds to TRPV1 receptors but with lower affinity and efficacy than to CB_1 cannabinoid receptors (Szallasi and Di Marzo, 2000). At a dose that had no effects on social play by itself, the TRPV1 vanilloid antagonist capsazepine did not block the reduction in social play induced by AM404, showing that this effect was not the result of stimulation of TRPV1 vanilloid receptors. The possibility that increased anandamide binding to either CB_1 or TRPV1 receptors as a result of treatment with AM404 reduces social play can also be ruled out, since the effect of AM404 persisted following combined pretreatment with SR141716A and capsazepine. Furthermore, neither the opioid receptor antagonist naloxone nor the dopamine receptor antagonist α-flupenthixol antagonized the reduction in social play induced by AM404. Thus, rather than being the consequence of increased endocannabinoid tone, we suggest that the reduction in social play induced by AM404 is due to effects on noncannabinoid targets. For example, it has been shown that AM404 inhibits Na^+ channels through non-CB_1, non-TRPV1 mechanisms (Nicholson et al., 2003; Kelley and Thayer, 2004). These off-target interactions complicate the interpretation of the behavioral effects of AM404 and indicate that AM404 may not be an optimal tool to study the effects of endocannabinoid transport inhibition (also see Cippitelli et al., 2007).

Because the effects of AM404 on social play were difficult to interpret pharmacologically, we tested the more selective anandamide uptake inhibitor VDM11 to characterize the effects of anandamide transporter inhibitors on social play behavior. VDM11 is an anandamide derivate that inhibits the anandamide transporter as potently as AM404, but it has no agonistic activity at TRPV1 receptors and is a weaker CB_1 receptor agonist than AM404 (De Petrocellis et al., 2000). At doses that did not alter social exploratory behavior or locomotor activity, VDM11 increased social play. This effect was mediated by activation of CB_1 cannabinoid receptors, since it was blocked by the CB_1 receptor antagonist SR141716A. Furthermore, the increase in social play induced by VDM11 was dependent on opioid and dopaminergic neurotransmission, since it was blocked by the opioid receptor antagonist naloxone and the dopamine receptor antagonist α-flupenthixol. Thus, the pharmacological profile of VDM11 in the modulation of social play is similar to that of the FAAH inhibitor URB597 (Trezza and Vanderschuren, 2008a), i.e., increased social play behavior that is dependent on cannabinoid, opioid, and dopaminergic neurotransmission. The present data therefore confirm and extend our previous findings (Trezza and Vanderschuren, 2008a,b), supporting the

notions that 1) endogenous anandamide plays an important role in the positive modulation of social behavior in adolescent rats; 2) cannabinoid-opioid interactions known to be involved in food and drug reinforcement also play an important role in the positive subjective properties of another natural reinforcer, i.e., social play behavior; and 3) the effects of indirect cannabinoid agonists on social play are dependent on dopaminergic neurotransmission, probably mediated through an endocannabinoid-induced increase in the activity of mesoaccumbens dopaminergic neurons.

Since endocannabinoids are synthesized and released on-demand following neuronal depolarization (Piomelli, 2003), inhibiting their deactivation prolongs their signaling in active synapses only, preserving the spatiotemporal specificity of endocannabinoid activity. The effects of the FAAH inhibitor URB597 (Trezza and Vanderschuren, 2008a,b) and the anandamide transporter inhibitor VDM11 (present study) therefore suggest that during social play, endocannabinoids are released in brain areas mediating this behavior. This endocannabinoid activity facilitates social play, so that URB597 and VDM11, by preventing anandamide hydrolysis and anandamide transport, respectively, enhance social play by magnifying endocannabinoid tone. In contrast, we have shown previously that stimulation of CB_1 cannabinoid receptors throughout the brain using the cannabinoid receptor agonist WIN55,212-2 reduced social play (Trezza and Vanderschuren, 2008a,b), perhaps by disrupting cognitive functions necessary to perform adequate social interactions (Egerton et al., 2006). In keeping with this finding, systemic administration of the stable analog of anandamide, (R)-methanandamide, reduced social play, through stimulation of CB_1 cannabinoid receptors, because the effect of (R)-methanandamide was blocked by the CB_1 cannabinoid receptor antagonist SR141716A. The latter finding also indicates that it is unlikely that increased anandamide levels as a result of anandamide transporter inhibition account for the effects of AM404 on social play. Rather, a non-CB_1 target seems to be responsible of the effects of AM404 on social play. Furthermore, these data support the hypothesis that the increase in social play induced by indirect cannabinoid agonists is due to enhanced anandamide signaling only in those brain areas mediating the positive subjective properties of social play behavior, rather than to increased anandamide signaling per se.

The molecular mechanism of anandamide uptake is a matter of debate. Since the anandamide transporter protein has not been cloned yet, its existence has been questioned and other mechanisms have been proposed, including FAAH-mediated simple diffusion (Glaser et al., 2003). However, genetic deletion of FAAH does not affect anandamide uptake, which argues against this possibility (Fegley et al., 2004). Furthermore, by using a selective ligand, a high-affinity anandamide transporter binding site distinct from FAAH has recently been identified (Moore et al., 2005). Together, these data suggest that the uptake and hydrolysis of anandamide are mediated by independent but functionally linked mechanisms.

VDM11 was originally found to selectively inhibit anandamide uptake (De Petrocellis et al., 2000). Studies into the effects of VDM11 on FAAH activity have yielded inconsistent results (Fowler et al., 2004; Vandevoorde and Fowler, 2005), which makes it unlikely that the increase in social play induced by VDM11 is the result of FAAH inhibition. Moreover, AM404, which is more potent than VDM11 in inhibiting

FAAH (De Petrocellis et al., 2000), reduced social play through noncannabinoid mechanisms, further indicating that FAAH is not the pharmacological target responsible for the opposite effects of AM404 and VDM11 on social play.

Our results, supporting an important role for anandamide in the modulation of social play behavior, suggest that anandamide transporter inhibitors might be useful for the treatment of neuropsychiatric disorders characterized by impairments in social behavior, and particularly by deficits in social play. For example, deficient cooperative play with peers and atypical social behavior are core symptoms of autism (Jordan, 2003), and lack of socialized patterns of play with subsequent social withdrawal are schizophrenic prodromal behaviors in children (Møller and Husby, 2000). Since social behaviors related and unrelated to play have different ontogenetic profiles and are mediated by dissociable neural systems (Vanderschuren et al., 1997), it is likely that the effects of indirect cannabinoid agonists on social behavior differ between young and adult individuals. Thus, further research into the effects of indirect cannabinoid agonists on social behavior is warranted. The present findings also suggest that target selectivity may account for the variability in the behavioral effects of these drugs, and they highlight the need to develop high-affinity ligands to exploit the therapeutic potential of endocannabinoid neurotransmission.

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